Endotoxin-Mediated Endothelial Cell Injury and Activation: Role of Soluble CD14

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Vascular endothelial cell (EC) injury by lipopolysaccharides (LPS) plays a major role in the pathogenesis of gram-negative bacterial sepsis and endotoxic shock. The studies described here were performed to define further the molecular mechanisms involved in the EC responses to LPS. We showed that serum was required for LPS-mediated cytotoxicity for bovine brain microvessel, pulmonary, and aortic ECs and that anti-human CD14 antibodies completely blocked LPS-mediated cytotoxicity for ECs in the presence of human serum. The addition of a recombinant soluble form of human CD14 to serum-free medium restored the LPS-mediated cytotoxicity, whereas the addition of LPS binding protein (LBP), a serum protein that potentiates LPS-induced responses to monocytes, had no effect. A similar dependency on serum or recombinant soluble CD14 (under serum-free conditions) was observed for LPS-induced secretion of interleukin-6 by human umbilical vein ECs. These findings indicate that soluble CD14 is required for LPS-mediated EC responses independently of LPB, suggesting that serum soluble CD14 represents a naturally occurring agonist for EC responses to LPS.

Bacterial lipopolysaccharides (LPS) or endotoxins are potent initiators of fever, coagulation disorders, multiple organ failures, and shock in humans and experimental animals (38, 40–42, 56, 63). Endothelial cell (EC) injury contributes significantly to the pathophysiology of gram-negative bacterial sepsis and endotoxic shock (13, 42). However, the mechanisms involved in LPS-induced EC injury are not well understood.

The biological activity of LPS is substantially modified by plasma proteins (4, 47, 51, 57, 61). Only recently have the mechanisms for LPS interactions with macrophages and monocytes been elucidated. These include a novel mechanism for LPS recognition that differs from the standard ligand-receptor model, in that it involves a plasma protein, LPS binding protein (LBP), which forms high-affinity complexes with LPS (47, 61, 62), and a plasma membrane glycoprotein, CD14. Upon exposure to plasma, LPS binds to LBP, leading to significantly enhanced LPS activity for the stimulation of macrophages and monocytes (37, 47, 61, 62). LBP is a glycoprotein that is present in normal adult plasma at 5 to 10 μg/ml but increases in concentration to 200 μg/ml after an acute-phase response (47, 52–54, 61, 62). CD14, a glycosylphosphatidylinositol-anchored protein expressed strongly on the surfaces of monocytes and macrophages and weakly on neutrophils (3, 9, 17, 21, 26), has been shown to be a receptor for LPS-LBP complexes (47, 61). Several investigations with isolated monocytes, neutrophils, whole blood ex vivo (10, 35, 47, 60, 61), or CD14-transfected 70Z/3 cells (33) have shown that the LBP-CD14-dependent pathway is closely linked to the initiation of cellular responses to LPS in these CD14-bearing cells. In contrast, binding studies with fluorescence-labelled anti-CD14 antibodies and fluorescence-activated cell sorter analysis failed to identify the presence of membrane CD14 in human umbilical vein ECs

(HUVEC) (7 and unpublished observations). Interestingly, while ECs do not express CD14 on their surface, they respond to LPS by expressing procoagulant activity (50) and adhesion molecules, such as ELAM-1 and ICAM-1 (8, 15), and by secreting various mediators, such as interleukin-1 (IL-1) and IL-6 (29, 34). Bovine ECs, in contrast to HUVEC, are exquisitely sensitive to LPS-mediated cytotoxicity (25, 39, 43). The responses of bovine and human ECs are serum dependent and may require a soluble form of CD14 (sCD14) found in serum, since anti-CD14 monoclonal antibodies (MAbs) can deplete the ability of serum to support LPS-induced EC activation or toxicity in vitro (19, 43). However, the importance of other serum factors, such as LBP, septin, or serum proteins yet undefined, involved in these responses cannot be ruled out (19).

In this study, we showed that purified recombinant sCD14, independently of other serum factors, can replace the requirement for serum in the responses of bovine ECs and HUVEC to both rough LPS and smooth LPS. While CD14 in monocyte and macrophage membranes plays a functional receptor role for LPS-LBP complexes, serum sCD14 appears to be essential for EC responses to LPS.

(This work was presented in part at the Second Conference of the International Endotoxin Society, Vienna, Austria, August 1992.)

MATERIALS AND METHODS

Reagents. Lipooligosaccharide (LOS) from *Haemophilus influenzae* type b DL42 (obtained from Eric Hansen, Dallas, Tex.) was isolated by the phenol-water method of Westphal et al. (58) and purified as described previously (59). *H. influenzae* type b LOS is a rough form of LPS. A smooth form of LPS was extracted from *Escherichia coli* 205 (O18 LPS; K nontypeable) by the hot phenol-water method of Westphal et al. (58) and purified as described previously (12). Fetal bovine serum (FBS) was purchased from GIBCO

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(Gaithersburg, Md.), rabbit serum was prepared by pooling platelet-poor sera from normal uninfected rabbits, and pooled normal human serum (PNHS) was derived from healthy adult laboratory workers. Murine anti-human CD14 MAbs 28C5 (immunoglobulin G2b [IgG2b]) (33) and My4 (IgG2b) (22, 24) were obtained from Didier Leturcq (R. W. Johnson Pharmaceutical Research Institute, La Jolla, Calif.) and Coulter Immunology (Hialeah, Fla.), respectively. A third anti-CD14 MAb, S39 (IgG2a), was also used (14, 22). An isotype-matched control MAb (MsIgG2b) was purchased from Coulter. Purified rabbit LBP and a polyclonal goat anti-rabbit LBP antibody (IgG) were obtained from P. S. Tobias (Scripps Research Institute, San Diego, Calif.), and LBP-immunodepleted rabbit serum was prepared as previously described (47). The biological activity of LBP was demonstrated by its ability to increase the amount of LPS-induced tumor necrosis factor alpha production in rabbit peritoneal exudate macrophages in the absence of serum as previously described (47).

sCD14. Recombinant sCD14 was expressed by use of the baculovirus expression system. In brief, a recombinant transfer vector containing human CD14 was constructed by ligating the blunt-ended human CD14 NcoI-MseI cDNA into the blunt-ended NheI site of the transfer vector, pBlueBac (Invitrogen). Sf9 insect cells were cotransfected with the recombinant transfer vector and wild-type Autographa californica nuclear polyhedrosis virus baculovirus DNA and grown in Sf900 medium (GIBCO-BRL) containing Bluo-Gal (150 μg/ml) (GIBCO-BRL). Recombinant plaques were identified by their blue color and were purified by routine methods (2). Under these conditions, human CD14 is expressed on the surface of the insect cells in a glycosylphosphatidylinositol-anchored form and in the medium as a soluble form (26a). For the production of large quantities of recombinant sCD14, insect cells were infected with the recombinant virus and grown in serum-free Sf900 medium. Recombinant sCD14 was isolated from the medium by affinity chromatography as described previously (23) and found to be >99% pure (by analysis of silver-stained sodium dodecyl sulfate-polyacrylamide gels).

ECs. Bovine brain cortical microvascular ECs (BBEC) were isolated by a modification of the method of Goetz et al. (20). On the basis of factor VIII antigen expression and the ability of cells to take up acetylated low-density lipoprotein, the resulting cell population was >95% ECs. Bovine pulmonary artery ECs (BPAE) and aortic ECs (BAE) were obtained from W. E. Laug (32) and maintained in Dulbecco modified Eagle-Ham F12 (DMEM-Ham F12) medium containing 20% FBS, penicillin (100 μg/ml), and streptomycin (100 μg/ml). BBEC were plated at 2×10^4 cells per 35-mm^2 well in 24-well culture plates (Costar) containing DMEM-Ham F12 medium. Cells reached confluency after 5 to 7 days in culture (approximate cell density, 3×10^5 cells per well). HUVEC were obtained from Clonetics (San Diego, Calif.) as cryopreserved primary cultures and passaged four times in bovine serum-containing medium in accordance with the manufacturer's instructions. HUVEC were >95% pure on the basis of factor VIII antigen expression.

LPS-mediated BBEC injury. BBEC were grown in 24-well culture plates (Costar). Confluent BBEC monolayers were washed three times in serum-free medium prior to the addition of LPS with or without serum. Cell injury was assessed as lactic dehydrogenase (LDH) release into the extracellular medium after 24 h of incubation by use of an LDH kit (228-UV; Sigma). For each experiment, quadruplicate control wells were used to measure spontaneous LDH

release (background) and total LDH after incubation of monolayers with 1% Triton X-100 for 15 min. Specific percent LDH release was measured as follows: [(test LDH − background)/(Triton X-100 LDH − background)] × 100.

IL-6 secretion by LPS-stimulated HUVEC. HUVEC were grown for 48 h in 96-well microtiter plates (seeding density, ≈4 × 10³ cells per well) containing growth medium supplemented with 10% FBS (GIBCO). HUVEC were washed four times in serum-free RPMI 1640 before the addition of different concentrations of LPS with and without 10% FBS or with sCD14 (1 μg/ml) in serum-free medium. After 6 h of incubation, the supernatants were harvested and assayed for IL-6 activity by use of the IL-6-dependent mouse hybridoma B9 cell line as previously described (1, 49).

RESULTS

LPS-mediated cytotoxicity for bovine ECs is serum dependent. Recent investigations suggested that the treatment of human serum with an anti-CD14 antibody depletes the serum-dependent response of bovine ECs to rough LPS (19). For determination of the effects of serum on the response to LPS, bovine EC (BBEC, BPAE, and BAE) monolayers were treated with increasing amounts of rough LPS and smooth LPS in the presence and absence of serum. Both forms of LPS induced cytotoxicity for ECs at doses as low as 10 ng/ml (Fig. 1). Serum was required for this LPS-mediated cytotoxicity, although very high concentrations of LPS (≥1 μg/ml) were able to induce some cytotoxicity in the absence of serum (Fig. 1). Heating the serum to 90°C but not to 56°C for 30 min abolished the EC injury (data not shown). LPS-mediated cytotoxicity was observed with sera from various species, including humans, fetal bovines, horses, pigs, and rabbits (data not shown). Serum concentrations as low as 0.5% (vol/vol) were sufficient to support LPS-induced EC injury.

LPS-induced cytotoxicity can be blocked by anti-CD14 antibodies in the presence of human sera. CD14 on the surface of monocytes and neutrophils acts as a receptor for LPS when LPS is complexed to serum LBP. sCD14 is found in normal serum (5, 30, 36). For further definition of the mechanisms of sCD14 involvement in LPS-mediated EC responses and for determination of the effects of various anti-CD14 antibodies on EC responses to both rough LPS and smooth LPS, BBEC monolayers were incubated in 10% FBS or 6% PNHS with anti-human CD14 MAbs, including My4, 28C5, and S39, all of which have been shown to inhibit monocyte- and macrophage- and/or CD14-bearing 70Z/3 cell responses to LPS (11, 28, 33). All three anti-CD14 MAbs at concentrations of ≥2.5 μg/ml provided complete protection against both rough LPS- and smooth LPS-induced cytotoxicity in the presence of human serum but not FBS (Fig. 2). The anti-CD14 MAbs also completely inhibited LPS-induced cytotoxicity for BPAE and BAE incubated with human serum (data not shown). An isotype-matched (IgG2b) control MAb (MsIgG2b) at concentrations as high as 20 μg/ml had no protective effect. These results suggest that CD14 plays an important role in the interaction of LPS with ECs. Unlike the results for the interaction of monocytes with LPS, increasing the LPS concentrations up to 1 μg/ml did not overcome the blocking effect of these anti-CD14 MAbs on EC cytotoxicity (data not shown).

For further investigation of the role of CD14 in LPS-EC interactions, we pretreated BBEC with 5 μg of My4 or 28C5 per ml, washed the cells, and incubated the monolayers with LPS in the presence of serum-containing medium. Cytotox-
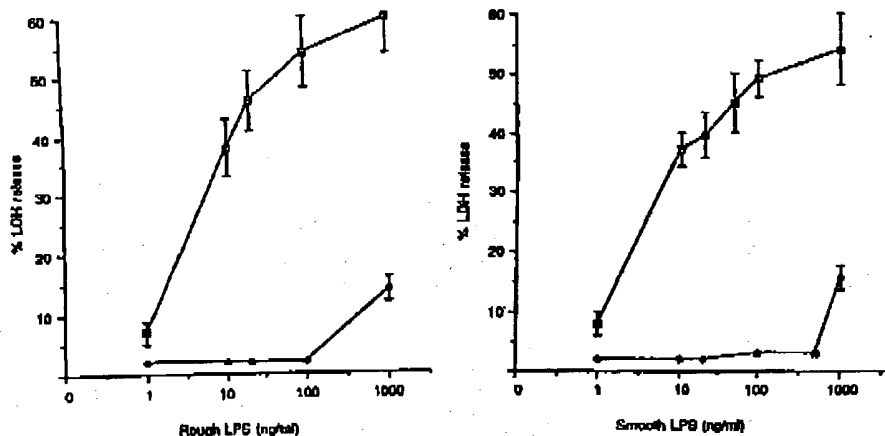
FIG. 1. Rough LPS and smooth LPS cause cytotoxicity to BBEC in a serum-dependent manner. BBEC monolayers were incubated with *H. influenzae* type b DL42 LOS (rough) or *E. coli* O18 LPS (smooth) in the presence (□) or absence (◆) of 10% FBS. Following overnight incubation, cell injury was measured as the specific percent LDH release into the extracellular medium as described in Materials and Methods. Values are averages for six samples ± standard deviations.

icity was not affected. This observation, together with the finding that the blocking effect of the anti-human CD14 MAbs was specific for human serum, suggests that anti-CD14 MAbs act by inhibiting the ability of a serum factor(s) to function in LPS-EC interactions rather than by blocking a cryptic form of membrane CD14 at the surface of ECs.

Exogenous LBP cannot restore LPS-induced cytotoxicity under serum-free conditions. LBP is a glycoprotein (60 kDa) that is present in normal plasma (47, 52, 61) and that binds with a high affinity to LPS (55). The interaction of LPS with LBP greatly increases the sensitivity of monocytes to LPS (47). Therefore, we investigated the role of purified LBP in LPS-EC-serum interactions. Confluent BBEC monolayers

were washed with RPMI 1640 and incubated in serum-free medium with LPS (20 ng/ml) in the presence or absence of rabbit LBP (bioactive across species [51a]). Smooth LPS or rough LPS was preincubated with LBP at various concentrations (LPS/LBP ratios of 1:5, 1:10, 1:20, and 1:100) for 30 min, and the mixture was then added to BBEC in serum-free medium. The addition of LPS-LBP complexes even at a ratio of 1:100 (i.e., 20 ng of LPS per ml and 2 μg of LBP per ml) did not induce any cytotoxicity (Fig. 3). In addition, when a polyclonal goat anti-rabbit LBP antibody was used to immunodeplete LBP from rabbit serum and BBEC monolayers were then incubated with LPS (100 ng/ml) in 10% normal rabbit serum or in 10% LBP-depleted rabbit serum, cytotox-
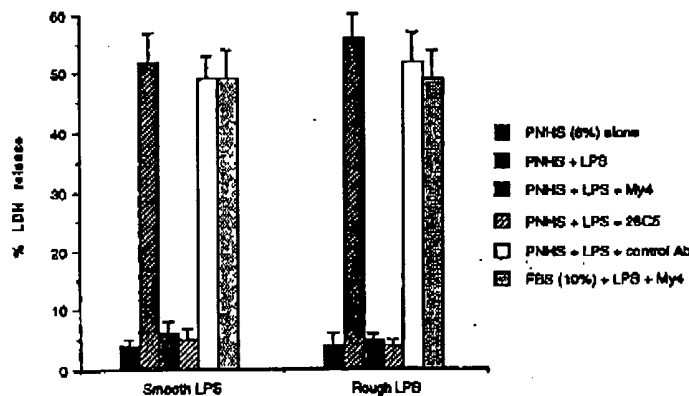

PNHS (6%) alone
PNHS + LPS
PNHS + LPS + My4
PNHS + LPS + 28C5
PNHS + LPS + control Ab
FBS (10%) + LPS + My4

FIG. 2. Anti-human CD14 MAbs block LPS-induced cytotoxicity in the presence of human sera. BBEC monolayers were incubated overnight with rough LPS or smooth LPS (100 ng/ml) in medium containing 6% PNHS, and LDH release was measured. Blocking anti-CD14 MAb My4 or 28C5 was added at 2.5 μg/ml, and an isotype-matched control MAb was added at 20 μg/ml as indicated, 30 min prior to the addition of LPS. MAbs against human CD14 were ineffective in the presence of 10% FBS, suggesting that these MAbs did not recognize bovine sCD14. Values are averages for six samples ± standard deviations.
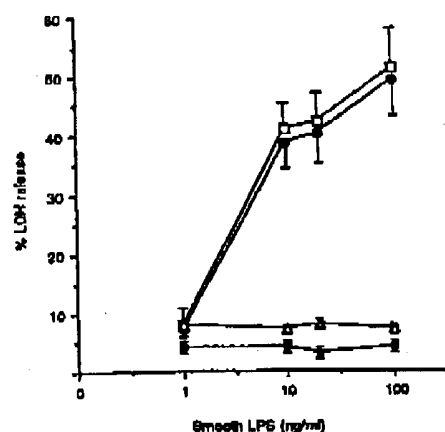
FIG. 3. The addition of recombinant human sCD14 replaces the serum requirement for LPS-mediated cytotoxicity. BBEC monolayers were incubated in medium containing 6% PNHS, with (■) or without (□) the addition of a blocking anti-CD14 MAb (My4) at 2.5 µg/ml as indicated and with smooth LPS (100 ng/ml). LDH release was measured following overnight incubation. BBEC monolayers were washed extensively, bathed in serum-free medium, and incubated with recombinant human sCD14 (1 µg/ml) (●) or purified rabbit LBP (2 µg/ml) (△) as indicated and with smooth LPS (100 ng/ml). The specific percent LDH release was measured following overnight incubation. Values are averages for four samples ± standard deviations. sCD14 alone up to 10 µg/ml did not cause detectable EC injury (data not shown).

FIG. 4. Dose-response experiments with exogenous sCD14 and the effect of LBP. BBEC monolayers were incubated in serum-free medium with E. coli O18 LPS (100 ng/ml), with increasing concentrations of exogenous recombinant sCD14, and with (■) or without (○) rabbit LBP (0.25 µg/ml). The specific percent LDH release was measured following overnight incubation. Values are averages for four samples ± standard deviations.

icity was observed for both normal rabbit serum and LBP-depleted rabbit serum (percent LDH release, 45 ± 5 and 37 ± 4, respectively; not significant). These observations indicate that LBP is not the serum factor required to support LPS-mediated EC injury.

sCD14 is the serum factor required to support LPS-induced EC injury. As mentioned above, in addition to membrane CD14, CD14 has been found in normal adult serum at concentrations between 2 and 4 µg/ml (5, 30, 36). For determination of whether sCD14 is the required serum factor that enables LPS-mediated EC injury, confluent BBEC monolayers were washed with RPMI 1640 and bathed in serum-free medium containing smooth LPS (100 ng/ml) and with or without exogenous recombinant human sCD14 (1 µg/ml). The addition of sCD14 restored LPS-mediated cytotoxicity for BBEC in the absence of serum (Fig. 3). In dose-response experiments, sCD14 concentrations as low as 0.1 µg/ml were sufficient to restore LPS-induced toxicity for BBEC (Fig. 4). These observations indicate that sCD14 is the serum factor required for LPS-mediated bovine EC toxicity. We next investigated the effects of purified rabbit LBP (0.25 µg/ml) on recombinant sCD14-dependent LPS-mediated EC cytotoxicity. The addition of exogenous LBP accelerated the appearance of visual cytopathic changes observed when BBEC were incubated with LPS (10 or 100 ng/ml) and sCD14; however, the specific percent LDH release at 24 h remained unchanged (Fig. 4).

In further experiments designed to investigate how sCD14 participates in LPS-mediated injury, preincubation of BBEC monolayers with recombinant sCD14 (up to 5 µg/ml, 60 min, 37°C), washing of the monolayers, and subsequent incuba-
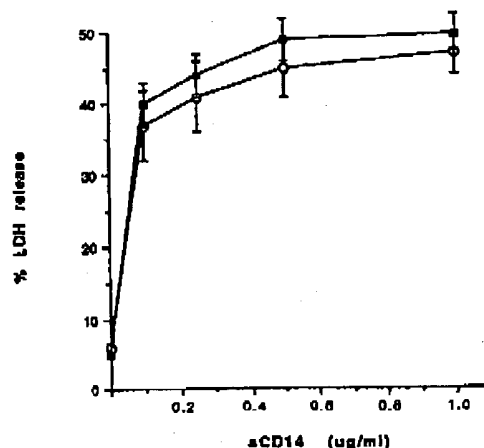
tion with LPS under serum-free conditions did not induce toxicity for these cells (Fig. 5). Similarly, preincubation of BBEC with smooth LPS in serum-free medium (100 ng/ml, 60 min, 37°C), washing of the monolayers, and subsequent incubation with medium containing 10% FBS or recombinant sCD14 (1 µg/ml) did not induce cytotoxicity (Fig. 5). These experiments suggested that sCD14 and LPS must be present simultaneously for the initiation of EC responses.

sCD14 is needed for IL-6 secretion from HUVEC. LPS has been shown to induce IL-6 secretion from HUVEC in serum-containing medium (29). Incubation of HUVEC in the presence or absence of serum showed that serum was required for the LPS induction of IL-6 secretion from these cells (Table 1). For investigation of whether sCD14 was involved in the serum-dependent LPS induction of IL-6 production, HUVEC grown in 96-well microtiter plates were incubated with smooth LPS (100 ng/ml) in the absence of serum and with or without recombinant sCD14 (1 µg/ml) for 6 h. The addition of exogenous sCD14 restored the response of the ECs to LPS (Table 1). Furthermore, an anti-CD14 MAb (My4) blocked LPS-induced IL-6 release from HUVEC incubated in medium containing PNHS (Table 1). On the other hand, the addition of LBP to serum-free medium had no effect (data not shown). Pretreatment of HUVEC monolayers with an anti-CD14 MAb (My4) had no effect on the ability of these cells to secrete IL-6 in response to LPS and serum. These data suggested that sCD14 in serum rather than cell-associated CD14 is necessary for HUVEC to respond to LPS.

DISCUSSION

Several recent investigations have shown that CD14, a membrane glycoprotein originally described as a differentiation antigen of monocytes, is a functional receptor for LPS-LBP complexes in monocytes and macrophages (47,
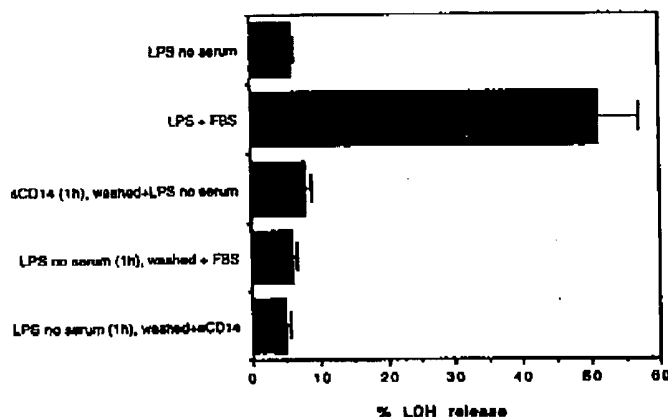
FIG. 5. EC injury mediated by LPS requires LPS and sCD14 to be present simultaneously. BBEC were incubated with sCD14 (1 μg/ml) in serum-free medium for 1 h at 37°C, and monolayers were washed with RPMI 1640 and then incubated with E. coli O18 LPS (100 ng/ml) in serum-free medium. In separate experiments, monolayers were incubated with O18 LPS (100 ng/ml) in serum-free medium for 1 h at 37°C, washed with RPMI 1640, and then incubated with medium containing 10% FBS or recombinant human sCD14 (1 μg/ml). The specific percent LDH release was measured following overnight incubation. Values are averages for three samples ± standard deviations.

61). This glycoprotein does not contain a transmembrane domain but is anchored to the cell surface by a phosphatidylinositol (PI) glycan anchor (26). In addition to membrane CD14, sCD14 has been observed in the serum of normal adults and in culture supernatants of CD14⁺ cells (5, 6, 26, 30). On the basis of recent studies, a model has been proposed in which LPS exposed to blood first interacts with the serum protein LBP and the resulting LPS-LBP complex then interacts with membrane CD14 to initiate LPS-induced responses in monocytes and macrophages and in neutrophils (35, 37, 47, 52-54, 60-62).

The vascular endothelium plays a major role in the pathogenesis of endotoxic shock and of blood-brain barrier damage in gram-negative meningitis (13, 42, 46). LPS can directly injure (25, 39) or activate ECs via a serum-dependent mechanism or through cytokines derived from activated monocytes and macrophages (44). Although a clear distinction exists between cell injury and activation, the functions displayed by activated ECs may lead to EC injury through indirect mechanisms (44). ECs lack surface CD14 expression, and the mechanisms of LPS-induced responses in these cells are less well understood than those in monocytes and macrophages. Recent investigations with affinity chromatography on an anti-CD14-Sepharose column showed that CD14-depleted serum failed to support a cytotoxic response of BPAE to rough LPS and that the addition of the eluted

material restored the response of the ECs to LPS (19). However, the importance of other serum factors involved in this response could not be ruled out by these experiments, as the authors concluded that it was not clear whether their sCD14 preparations (the eluted material) were contaminated with LBP, septin, or other proteins (19). In this study, we investigated the mechanisms involved in LPS-mediated bovine EC injury and LPS-induced IL-6 secretion by HUVEC and the role of serum proteins, such as sCD14 or LBP, by using purified proteins in serum-free experiments.

In accordance with recent studies (19, 43), we observed that both rough LPS and smooth LPS caused dose-dependent toxicity for BBEC, BAE, and BPAE. We also observed that serum was absolutely required and that the cytotoxicity was completely blocked by anti-CD14 MAbs in the presence of human sera. Serum was also required to activate HUVEC with LPS to produce IL-6 secretion. In addition, we demonstrated that recombinant human sCD14 successfully replaced the serum requirement for both of these LPS-induced responses. These findings suggest that sCD14 is a novel agonist that is required for mediating serum-dependent LPS responses in ECs. This mechanism may be an effective pathway for LPS interactions with cells, such as ECs, that do not express surface CD14.

Normal adult serum contains relatively high concentrations of sCD14 (2 to 4 μg/ml). Although monocytes can shed

TABLE 1. Effects of serum and sCD14 on LPS-induced IL-6 secretion from HUVEC

LPS (ng/ml)	IL-6 release (pg/ml)[a] in the presence of:			
	6% FNHS	No serum	No serum + sCD14	6% FNHS + My[a]
10	254 ± 25	35 ± 7	240 ± 20	44 ± 7
20	312 ± 32	38 ± 8	285 ± 32	62 ± 5
100	408 ± 57	40 ± 10	350 ± 44	77 ± 8

[a] HUVEC were incubated with various concentrations of E. coli LPS for 6 h in the presence or absence of FNHS, with or without an anti-CD14 MAb (My4; 2.5 μg/ml), and with recombinant human sCD14 (1 μg/ml) as indicated, and IL-6 levels were measured in the supernatants. Values are averages for four samples ± standard deviations.